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EKLF restricts megakaryocytic differentiation at the benefit of erythrocytic differentiation

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Abstract

Previous observations suggested that functional antagonism between FLI-1 and EKLF might be involved in the commitment toward erythrocytic or megakaryocytic differentiation. We show here using inducible shRNA expression, that EKLF knockdown in MEL cells decreases erythrocytic and increases megakaryocytic as well as *Fli-1* gene expression. Chromatin immunoprecipitation analyses revealed that the increase in megakaryocytic gene expression is associated with a marked increase in RNA pol II and FLI-1 occupancy at their promoters albeit FLI-1 protein levels are only minimally affected. Similarly, we show that human $CD34^+$ progenitors infected with shRNA lentivirus allowing EKLF knockdown generate an increased number of differentiated megakaryocytic cells associated with increased levels of megakaryocytic and *Fli-1* gene transcripts. Single cell progeny analysis of a cell population enriched in bipotent progenitors revealed that EKLF knockdown increases the number of megakaryocytic at the expense of erythrocytic colonies. Taken together, these data indicate that EKLF restricts megakaryocytic differentiation at the benefit of erythrocytic differentiation and suggest that this might be at least partially mediated by the inhibition of FLI-1 recruitment to megakaryocytic and *Fli-1* gene promoters.

Introduction

Erythrocytic and megakaryocytic lineages derive from a common bipotent progenitor (MEP) able to generate only erythrocytic or megakaryocytic progenitors [1,2]. A common bipotent precursor (PEM) has also been characterized in the spleen of anemic mice [3,4]. Despite this very close proximity, the molecular mechanisms controlling commitment toward either one of these two lineages remain poorly understood.

Two main models have been proposed to explain the commitment of multipotent hematopoietic progenitors [5,6]. According to the "instructive model", lineage commitment is dictated by specific extracellular signals such as cytokines. Although erythropoietin and thrombopoietin enhance the proliferation, survival and differentiation of already committed erythrocytic and megakaryocytic progenitors expressing erythropoietin or thrombopoietin specific receptors, it is now well established that they have no instructive role in the commitment [7,8].

Available data are more compatible with a "stochastic model" suggesting that commitment is dictated by the spontaneous formation of specific and mutually exclusive combinations of transcription factors [5,6]. At least ten different transcription factors involved in erythrocytic and/or megakaryocytic differentiation have been identified [9-12]. Most of them, like GATA-1, NF-E2, TAL-1, LMO2, FOG1 and GFI-1B are involved in the regulation of both erythrocytic and megakaryocytic differentiation. However, there is no indication that differential expression of either one of these six factors might be involved in the commitment decision. Several transgenic mice carrying different knockdown mutations of *c-myb* gene display reduced levels of erythrocytic and increased levels of megakaryocytic differentiation [13,14] but the C-MYB target genes responsible for this differential effect remain largely unknown [15]. Three other transcription factors, RUNX1, FLI-1 and EKLF display clear differential expression, RUNX1 and FLI-1 being specifically expressed in megakaryocytic cells [16] and EKLF in erythrocytic cells [17]. Conditional inactivation of *runx1* in adult mice inhibits megakaryocytic differentiation but does not increase erythrocytic differentiation [17-19]. In contrast to RUNX1, several data argue for a role of FLI-1 and EKLF in the commitment decision between erythrocytic *versus* megakaryocytic differentiation [20]. Many megakaryocytic gene promoters are characterized by conserved functional GATA and ETS binding sites [9] and most of them are directly activated by FLI-1 [21-23]. For example, FLI-1 and

GATA-1 interact together and cooperate with FOG1 to activate the *GpIX* gene through their cooperative binding to its promoter [22,24]. In agreement to these data, homozygous *Fli-1* gene deletion leads to megakaryopoiesis defects in mice embryos [21,25,26] whereas hemizygous loss of *Fli-1* is responsible for dysmegakaryopoiesis in the human Paris-Trousseau syndrome [27,28]. Contrasting with this role of FLI-1 in the positive control of megakaryopoiesis, enforced expression of FLI-1 blocks the erythrocytic differentiation of normal avian progenitors as well as several human or murine erythroleukemic cells [29-32]. Furthermore, a recent study showed that ES cells contribution to erythroid progenitors of chimeric mice is greatly enhanced when using *Fli-1* +/- instead of *Fli-1* +/+ cells [33].

Reciprocally, several erythrocytic gene regulatory regions are characterized by tandem GATA-1 and EKLF binding sites. Knockout experiments have shown that EKLF is involved in the activation of many erythrocytic genes although erythrocytic progenitors can be produced in the absence of EKLF [17,34-40]. Contrasting with this positive role of EKLF in erythropoiesis, transgenic mice overexpressing EKLF under the control of LCR and β-globin gene promoter display an unexplained reduction of platelets number [41]. Furthermore, transcriptome analyses of *Eklf* -/- mouse fetal livers revealed increased levels of several megakaryocytic gene transcripts [17]. In that context, we recently demonstrated functional antagonism between FLI-1 and EKLF in transient expression assays [20,39]. All these data led us to suggest that functional antagonism between FLI-1 and EKLF might be involved in the commitment toward erythrocytic or megakaryocytic differentiation but this hypothesis still remained to be demonstrated.

The present study combined inducible and constitutive shRNA expression to knockdown EKLF in MEL cells and in normal human progenitors. Concordant results of these experiments demonstrate that EKLF actually restricts megakaryocytic at the benefit of erythrocytic gene transcription and differentiation and suggest that this negative effect of EKLF on megakaryocytic commitment is mediated at least partially through the inhibition of FLI-1 recruitment to megakaryocytic as well as to *Fli-1* gene promoters.

Material and method

Cell lines culture and transfection

All cell lines (MEL 745-A, K562) were grown in Iscove modified Dubelcco's medium (Invitrogen, France) supplemented with 10% fetal calf serum (Biowest, France) and penicillin-streptomycin. Clone 745/TR has been derived from 745-A cells following

transfection with plasmid pEF1α-TetR expressing the bacterial repressor TetR and blasticidin resistance [42,43]. Clones 4D7 and 2M12 were derived from 745/TR cells following transfection with pGJ10/shEKLF-1 or pGJ10/shEKLF-2 plasmids encoding two different shRNA directed against *Eklf* mRNA under the control of a doxycycline inducible H1 promoter [42,44] and G418 resistance. Clone 745/TR was maintained under constant selection with blasticidin (20 μg/mL) whereas clones 4D7 and 2M12 were maintained in blasticidin (20 μg/mL) (Cayla, France) and G418 (1 mg/mL)(Invitrogen, France). Differentiation of 4D7 and 2M12 cells was induced by adding 5 mM HMBA (hexamethylenebisacetamide, Sigma) and induction of shRNA production by adding 2 μg/mL of doxycycline (Clonetech, France). Transfection of K562 cells was performed by electroporation using Nucleofector kit V and program T-16 of the Amaxa Electroporator (Nucleofector, Amaxa, France). Transfection of MEL cells was performed using DAC30 (Eurogentech, Belgium) according to the manufacturer's instructions.

CD34$^+$ purification and culture

Leukocytes were obtained from cord blood by ficoll gradient concentration. CD34$^+$ cells were then selected using a magnetic cell sorting system (miniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's recommendations. The purity of recovered cells was checked by FACS analysis and always found superior to 80%. In most experiments, purified CD34$^+$ cells were cultured in serum-free medium [45] supplemented with recombinant human thrombopoietin (r-huTPO, 10 ng/mL), stem cell factor (r-huSCF, 25 ng/mL) and erythropoietin (r-huEPO, 1 U/mL) (E/MK medium). Recombinant cytokines were obtained from Amgen Corp (Thousand Oaks, CA, USA) except for r-huTPO which was a gift from Kirin Brewery (Tokyo, Japan).

Plasmid constructs

pSG5, pSG5 HA-EKLF [20], pEF, pEF FLI-1 [20], pEF1α-TetR [42] , pGJ10[43], pH1 and pRRL [46] have been already described. p668 (kindly provided by Dr J Bieker) has been generated by subcloning murine EKLF cDNA upstream of the IRES-GFP sequence of the MSCV-based MiG retrovirus [47]. pSG5 HA-EKLF/M-Zn1-3 has been generated by subcloning the mutated EKLF region of pSG5 EKLF/M-Zn1-3[48] into pSG5 HA-EKLF. pGJ10/shEKLF-1 and pGJ10/shEKLF-2 were obtained by cloning double stranded oligonucleotides encoding shRNA directed against two different target sites of the murine *Eklf* gene into Bgl II and Not I

sites of pGJ10. Similarly, double stranded oligonucleotides encoding scramble shRNA or shRNA directed against human *Eklf* mRNA were first cloned downstream to the H1 promoter of pH1 vector. The pH1 promoter and shRNA cassettes were then isolated by Xho1 digestion and cloned into the single Xho1 site of pRRL leading to SCR shRNA and *Eklf* shRNA pRRL lentiviral vectors. Sequences of oligonucleotides used for these constructs are given on supplementary Table S1. Sequences of all shRNA coding regions were verified by direct sequencing of final DNA constructs.

Lentivirus production and cells infection

Lentivirus supernatants were prepared by cotransfection of 293-T cells with pRRL plasmids (pRRL SCR shRNA or pRRL *Eklf* shRNA), the packaging plasmid pCMV R874 and the VSV-G protein envelope plasmid pMDG as previously described [46]. $CD34^+$ cells (1 to 3 10^5 cells/mL) were cultured up to 3 days in serum-free medium supplemented with TPO, EPO, SCF (E/MK medium). The third day, cells were centrifuged, resuspended (3 10^6 cells/mL) in fresh cytokine-supplemented medium containing 2 µg/mL hexadimethrine bromide (Sigma, France) and exposed to lentiviral supernatants during 3 hours at 37°C. Immediately following infection, cells were washed in phosphate buffered saline (PBS) and reseeded in E/MK medium either directly or after sorting of single $CD36^+CD31^{Med}$ cells by FACS (see Supplementary Figure 1). In order to favor erythroid differentiation in the experiments presented in Figure 5, $CD34^+$ cells were cultured in the same medium but with IL3 (r-huIL3, 100 U/mL) instead of TPO and supplemented with 20% FCS for 3 days, infected for 1 day, reseeded for 2 days in the same medium and then for 1 day in medium supplemented with 30 % FCS before the sorting of infected erythrocytic differentiated cells (GFP^+CD36^+, GPA^+,$CD41^-$).

Transcript analyses

Total cell RNA was prepared by RNA plus (Q-biogene, France). Total RNA (1 µg) was reverse transcribed using 200 U Moloney reverse transcriptase (Invitrogen) and random oligonucleotide hexamers in a final volume of 20 µL during 1 hour at 37°C. 1 µL of reverse transcription reaction was then amplified by qPCR using SYBR Green PCR kits (Qiagen or Roche, France) and a Lightcycler (Roche, France). Primers sequences used for qPCR are given in supplementary Table S1.

Western blot analyses

Western blot analyses were performed on total cell lysates as previously described [20] using the following antibodies : anti-EKLF (Santa Cruz, sc-27194)(Figure 4), anti-EKLF monoclonal (clone 6B3)[48] (Figure 2), anti-GRB-2 (Santa Cruz, sc-17813), anti-FLI-1 (Santa Cruz, sc-356 or ABcam, Ab15-289-500), anti-GATA-1 (Santa Cruz, sc-1233), anti-HSC70 (Sressgene, SPA-815).

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed following the Upstate's protocol with minor modifications. Briefly, 10^8 cells were fixed for 10 min at room temperature by adding $1/10^{th}$ volume of a 10X fixation solution (PBS 1X, 11% formaldehyde, 0.1 M NaCl, 50 mM HEPES pH 7.9) directly to the culture medium. Fixation was stopped by adding 0.125 M glycine followed by two washes in cold PBS. Chromatin was prepared by incubating fixed cells (5 10^7 cells/mL) for 10 min on ice in lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA, 1% SDS, containing $1/25^{th}$ volume of EDTA free protease inhibitors cocktail (Roche)). Chromatin fragmentation was performed on ice by 5-10 pulses of sonication (Bioblock Vibra Cell 72405 sonicator). Aliquots of chromatin were taken at this step to control the mean length of fragmented DNA by agarose gel electrophoresis (less than 500 bp) and to quantify the DNA concentration by quantitative PCR. Sonicated chromatin preparations (corresponding to 2 10^7 cells) were first diluted in 10 volumes of ChIP IP buffer (16.7 mM Tris pH 8, 1.2 mM EDTA, 167 mM NaCl 1.1 %, Triton X100, 0.01 % SDS containing $1/20^{th}$ volume of protease inhibitors cocktail (Roche)) and pre-cleared by adding 100 µL of protein G sepharose beads (Amersham) for 4 hours at 4°C on a rolling wheel. Pre-clearing beads were eliminated by centrifugation and immunoprecipitations were performed overnight at 4°C on a rolling wheel by adding 2 µg of antibody (antibodies used : anti-FLI-1 (Santa Cruz, sc-356), anti-GATA-1 (Santa Cruz, sc-256), anti-RNA-polymerase II (Santa Cruz, sc-899), anti-acetylated Histone H3 (Upstate, 06-599)). Protein G sepharose beads were added (10 µL for each mL of immunoprecipitation starting volume) and incubation was pursued for 3 hours at 4°C. Beads were washed 5 times (5 min each at 4°C) in 1 mL of the following buffers : once in W1 (20 mM Tris pH 8, 2 mM EDTA, 150 mM NaCl, 0.1 % SDS, 1 % Triton X100), once in W2 (20 mM Tris pH 8, 2 mM EDTA, 500 mM NaCl, 0.1 % SDS, 1 % Triton X100), once in W3 (10 mM Tris pH 8, 1 mM EDTA, 1 % NP40, 1 % sodium deoxycholate, 0.25 M LiCl) and twice in W4 (10 mM Tris pH 8, 1 mM EDTA). Chromatins were eluted in a final volume of 500 µL by two successive

incubations of the beads (15 min at room temperature) in 250 µL of elution buffer (0.1 M $NaHCO_3$, 1 % SDS) and treated with proteinase K. Crosslink was reversed by overnight incubation at 65°C and DNA was finally prepared by classical phenol/chloroform extraction and ethanol precipitation. Immunoprecipitated and input DNA (prepared from aliquots taken before the immunoprecipitation step) were quantified by real time PCR using SYBR green PCR kits. Primer sequences used for qPCR are given in supplementary Table S1.

FACS analysis and sorting

Phycoerythrin (PE)-conjugated anti-CD31, anti-CD42 and anti-GPA; allophycocyanin (APC)-conjugated anti-CD41 and anti-CD36; PE- and APC-conjugated IgG_1 and IgG_{2a} controls MoAbs were from BD Biosciences (Le Pont de Claix, France). Cells were suspended in PBS, stained for 30 minutes at 4°C in accordance with manufacturer's recommendations and either analyzed on a FACsort® with the Cell Quest software package or sorted on a FacsDIVA flow cytometer equipped with an automatic cell deposition unit (BD Biosciences).

Results

Endogenous EKLF represses megakaryocytic genes expression during erythroid differentiation of MEL cells

As it could be predicted from our previous studies [20], we found that enforced EKLF expression is indeed able to antagonize FLI-1 induced expression of endogenous *GpIIIa* and *GpIX* megakaryocytic genes in K562 cells (supplementary Figure S3). This prompted us to investigate if endogenous EKLF could repress megakaryocytic genes during erythroid differentiation. For that purpose, we decided to knockdown EKLF during terminal erythroid differentiation of MEL cells (clone 745-A) using inducible shRNA expression. We thus established several clones of 745-A cells allowing doxycycline-inducible production of shRNA directed against two different regions of *Eklf* mRNA. Clones 4D7 (target 1) and 2M12 (target 2) which displayed the highest reduction of EKLF protein in the presence of doxycycline were selected for further analyses. In the experiments presented in Figure 1, 4D7 and 2M12 cells were grown for two days in the presence of doxycycline to induce EKLF knockdown and then for two days (still with or without doxycycline) in the presence of HMBA to induce erythroid differentiation. In both clones, clear reduction of EKLF protein

was observed in the presence of doxycycline (Figure 1A; lanes 1 and 2). As expected, *β–globin* and *AHSP* (alpha hemoglobin stabilizing protein) mRNA, two well known erythroid direct target genes activated by EKLF [17,39,49], were significantly reduced when differentiation was induced in the presence of doxycycline (Figure 1B, lanes 2 and 3). In marked contrast, doxycycline induced a significant increase in the mRNA levels of the three megakaryocytic genes, *Gp1b, GpIX* and *GpIIIa* as well as *Fli-1* genes (Figure 1B; lanes 4- 7). These results thus established that endogenous EKLF actively contributes to repress megakaryocytic and *Fli-1* gene expression during erythroid differentiation of MEL cells.

EKLF knockdown stimulates the recruitment of RNA polymerase II and FLI-1 to megakaryocytic and *Fli-1* gene promoters

To determine the mechanisms which could explain megakaryocytic genes repression by endogenous EKLF in MEL cells, 4D7 cells were grown for two days with or without doxycycline and for further two days with HMBA as in Figure 1. Then, we used chromatin immunoprecipitation (ChIP) followed by quantitative PCR to determine RNA polymerase II occupancy at the *Gp1b* and *GpIX* gene promoters (Figure 2). ChIP experiments performed concurrently using anti-HA antibody and quantitative PCR of the silent *myoD* gene were used as negative controls. As expected, no significant RNA pol II occupancy was found at the silent *myoD* gene sequence (Figure 2A; lanes 1, 2 and 7, 8). In contrast, significant RNA pol II occupancy was found at the *GpIX* and *Gp1b* promoters in the absence of doxycycline (Figure 2A; compare lanes 9, 11 to lane 7). RNA pol II occupancy at the *GpIX* and *Gp1b* promoters increased by two fold in the presence of doxycycline thus indicating enhanced transcription. Similarly, when compared to *myoD*, the *GpIX* and *Gp1b* promoters already displayed high levels of acetylated histone H3 whereas these levels slightly increased after EKLF knockdown (Figure 2B). According to previous studies, the transcription of *GpIX* and *Gp1b* genes is critically dependent on FLI-1 and GATA-1 recruitment to their promoters [23,50]. Intriguingly, however, FLI-1 and GATA-1 protein levels were only minimally increased by EKLF knockdown (+20% or +10% respectively, see insert in Figure 3A). This prompted us to investigate the effect of EKLF knockdown on FLI-1 and GATA-1 occupancy at the *GpIX* and *Gp1b* promoters. As above, HA antibody and the *myoD* gene which is devoid of GATA-1 and FLI-1 binding sites were used as negative controls. In the absence of doxycycline, significant occupancy of both FLI-1 and GATA-1 (2 or 3 fold above background; compare lane 19 to lanes 13, 17 and 21 in Figure 3A and Figure 3B respectively) was already detected at the *Gp1b* promoter, whereas no significant occupancy was detected at the *GpIX* promoter (Figure

3A and 3B, compare lane 15 to lanes 13, 17 and 21). In the presence of doxycycline, both FLI-1 and GATA-1 occupancy increased at the *Gp1b* promoter (3 and 1.5 fold respectively; Figure 3A and 3B compare lanes 19 and 20) whereas FLI-1 occupancy increased by 2 fold and GATA-1 remained undetectable at the *GpIX* promoter (Figure 3A and 3B respectively, compare lanes 15 and 16). These data thus indicate that transcriptional activation of *GpIX* and *Gp1b* genes induced by EKLF knockdown is associated with increased recruitment of FLI-1 to their promoters as well as with increased recruitment of GATA-1 to the *Gp1b* promoter. Interestingly enough, FLI-1 occupancy was also increased at the *Fli-1* gene promoter itself (Figure 3A, compare lanes 23 and 24).

EKLF knockdown in human cord blood progenitors favors their megakaryocytic differentiation at the expense of erythrocytic differentiation

The above results indicating that EKLF represses megakaryocytic gene expression in MEL cells prompted us to investigate the effect of EKLF knockdown on the *in vitro* differentiation of normal human primary progenitors. For that purpose, purified $CD34^+$ human cord blood progenitors were first amplified in serum free medium containing EPO, TPO and SCF (E/MK medium) favoring the growth and differentiation of erythrocytic and megakaryocytic progenitors for three days and infected for 3 hours with lentiviruses allowing constitutive expression of GFP and shRNA directed against *Eklf* mRNA or control shRNA of scrambled sequence (SCR). Infected progenitors were then reseeded in E/MK medium and their cell progeny analyzed 7 days latter. In three independent experiments, unsorted progenitors transduced with *Eklf* or control shRNA lentiviruses always generated the same number of cells and the same high percentage of GFP^+ cells (>80%) thus indicating high and similar infection efficiencies. In these studies, *Eklf* shRNA reduced EKLF protein expression by more than 85% in GFP^+ cells (Figure 4A). Most of the GFP^+ cell population remained mainly composed of erythrocytic GPA^+ cells but these erythrocytic cells expressed lower levels of GPA (Figure 4B). A similar decrease in GPA expression was observed at the transcriptional level in previous studies of murine *Eklf* -/- fetal liver cells[17]. The percentages of $GFP^+CD41^+CD42^+$ megakaryocytic cells were found to vary between different preparations of $CD34^+$ progenitors. However, for each given experiment performed with the same batch of $CD34^+$ cells, the percentage of $GFP^+CD41^+CD42^+$ megakaryocytic cells was systematically and significantly increased (mean fold increase 2.8; p value = 0.05) in cells transduced with the *Eklf* shRNA lentivirus (Figure 4C). In agreement with these data, cells transduced with the *Eklf* shRNA lentivirus displayed a marked decrease in *Eklf* and *Gpa*

mRNA levels and a reciprocal increase in *GpIX* and *GpIIIa* mRNA (Figure 4D). Moreover, *Fli-1* mRNA level was also increased by EKLF knockdown. Taken together, these results indicated that EKLF knockdown led a two to three fold increase in the megakaryocytic differentiation output of cord blood progenitors associated with increased expression of megakaryocytic and *Fli-1* genes and decreased expression of the known EKLF target gene *Gpa* in erythroid cells.

Although the increase in megakaryocytic gene expression roughly corresponded to the increase in megakaryocytic cells number, we wanted to determine if EKLF knockdown could also induce megakaryocytic gene expression in already committed erythrocytic cells. To address this question directly, $CD34^+$ progenitors were grown for three days in conditions favoring erythroid differentiation (EPO, IL3, SCF) and infected with either control or *Eklf* shRNA lentivirus. Infected late erythrocytic cells ($GFP^+CD36^+GPA^+CD41^-$) were then purified by FACS for transcript analyses three days later. As above, analysis of the whole cell population transduced with *Eklf* shRNA lentivirus revealed a marked decrease in EKLF protein (Figure 5A) as well as, *Eklf*, *Gpa* and *β-globin* mRNA (Figure 5B, lanes 1 and 2). A concomitant increase in megakaryocytic *GpIIIa* and *GpIX* mRNA levels was also observed but these levels were at least ten fold lower than that observed in the previous experiments performed in culture conditions favoring both erythrocytic and megakaryocytic differentiation (Figure 5B; lanes 1 and 2). As expected, a similar reduction of *Eklf, Gpa and β-globin* mRNA was observed in late erythrocytic cells (Figure 5B; lanes 3, 4). In contrast, *GpIIIa* and *GpIX* mRNA in late erythrocytic cells remained almost undetectable even after infection with *Eklf* shRNA lentivirus. Taken together, these results indicated that EKLF knockdown led a two to three fold increase in the megakaryocytic differentiation output of cord blood progenitors without inducing any detectable reactivation of megakaryocytic genes in fully committed erythrocytic cells.

Several mechanisms could explain the increase in megakaryocytic output of $CD34^+$ progenitors following EKLF knockdown including selective outgrowth of megakaryocytic progenitors, selective loss of erythrocytic progenitors or redirection of more immature erythrocytic and/or bipotent progenitors towards megakaryocytic differentiation. To distinguish between these possibilities, we decided to analyze the single cell progeny of a sorted infected cell population enriched in bipotent progenitors. As above, purified $CD34^+$ human cord blood progenitors were first amplified for three days in E/MK medium, infected for 3 hours with *Eklf* or control shRNA lentiviruses. Immediately following infection, $CD31^{Med}CD36^+$ cells which were found to be enriched in erythro-megakaryocytic bipotent

progenitors (see below) were sorted by FACS and reseed at one cell per well for 7 days in E/MK medium. The use of short infection time was justified to minimize any bias due to preferential cell proliferation, survival or death which could occur before the sorting of single $CD31^{Med}CD36^{+}$ cells. Since this infection time was too short to allow detectable expression of GFP for the sorting of infected cells, infected GFP^{+} cells were identified *a posteriori* at the time of the phenotypic analyses 7 days post-infection. Three clearly distinct types of GFP^{+} clones could be identified : erythrocytic clones (containing large number of small cells expressing GPA), megakaryocytic clones (containing small numbers of large cells expressing CD41) and mixed erythro-megakaryocytic clones (containing intermediate numbers of small and large cells expressing GPA or CD41) (Figure 6A). The presence of mixed clones as well as the high cloning efficiency of this $CD31^{Med}CD36^{+}$ population attested its enrichment in erythro-megakaryocytic bipotent progenitors. Interestingly, the percentage of GFP^{+} megakaryocytic clones derived from cells infected with *Eklf* shRNA lentivirus was significantly increased and that of erythrocytic clones was significantly decreased compared to cells infected with control shRNA lentivirus. Concomitantly, the percentage of mixed clones also decreased although the decrease was not statistically significant (Figure 6B). Importantly, the overall cloning efficiency in each experiment did not differ between cells infected with *Eklf* or control shRNA lentivirus (54 % *vs* 55 %, 88 % *vs* 84 % and 82 % *vs* 84 % respectively for the three experiments performed). Altogether, these results indicated that the enhanced megakaryocytic differentiation output induced by EKLF knockdown occurred at the expense of erythrocytic differentiation without selective out-growth or loss of initial clonogenic progenitors. This in turn strongly suggested that EKLF knockdown increased the commitment of bipotent progenitors toward megakaryocytic differentiation at the expense of erythrocytic differentiation.

Discussion

One of the main conclusion of this study is that endogenous EKLF restricts the commitment of human bipotent progenitors toward megakaryocytic differentiation at the benefit of erythrocytic differentiation. This conclusion is supported by concordant observations showing that shRNA mediated EKLF knockdown increases the in vitro production of megakaryocytic cells by $CD34^{+}$ multipotent progenitors and most importantly increases the relative proportion of megakaryocytic *versus* erythrocytic colonies generated by bipotent progenitors. Two crucial points must be taken into account in the interpretation of these results. First, single cell sorting of the $CD31^{Med}CD36^{+}$ bipotent population has been

done only three hours after the beginning of the infection with lentivirus expressing shRNA. This very short time excludes any bias which could be due to differential growth of erythrocytic or megakaryocytic progenitors during infection. Furthermore, the cells infected with either *Eklf* or control shRNA lentivirus displayed the same cloning efficiency thus excluding any bias which could be due to preferential death of erythrocytic progenitors and/or preferential survival of megakaryocytic progenitors with reduced EKLF.

This study also shows that EKLF displays the interesting property to repress FLI-1-dependent endogenous megakaryocytic gene transcription. Indeed, enforced expression of EKLF represses FLI-1-induced expression of endogenous megakaryocytic genes in K562 cells (Figure S3). Reciprocally, in both MEL cells and human $CD34^+$ progenitors, EKLF knockdown not only leads to an expected decrease in the expression of its known target genes (such as *Gpa*, *Ahsp* or *β-globin*) but also to a concomitant increase in the expression of several megakaryocytic genes (*GpIX, Gp1b* or *GpIIIa*) already known as direct target genes of FLI-1, including the *Fli-1* gene itself. Most importantly, the increase in megakaryocytic and *Fli-1* gene expression in MEL cells is associated with a marked increase in FLI-1 protein occupancy at their promoters. These observations strongly suggest that the repression of megakaryocytic genes by endogenous EKLF is at least partially dependent on the direct or indirect repression of *Fli-1* gene expression and auto-activation. This interpretation is in agreement with our failure to get any evidence for a role of endogenous EKLF in the repression of megakaryocytic genes in fully committed erythrocytic cells which do not express FLI-1. Indeed, we did not find any re-expression of megakaryocytic genes in purified erythrocytic cells generated from $CD34^+$ progenitors after EKLF knockdown although, in the same experiment, EKLF knockdown was clearly sufficient to decrease the expression of EKLF target genes (*Gpa* and *β-globin*). This suggests in turn that endogenous EKLF most probably only initiates the repression of megakaryocytic genes in bipotent progenitors still expressing FLI-1. In that respect, it would be interesting to know if the lack of megakaryocytic gene expression in fully committed erythrocytic cells is only due to the loss of FLI-1 expression or if others mechanisms (such as chromatin condensation for example) are also involved. Interestingly enough, we recently found, using transgenic mice allowing inducible deletion of the *Fli-1* gene, that FLI-1 is absolutely required for the megakaryocytic commitment of bipotent MEP (Joëlle Starck and FM unpublished results 2007). All together, these data strongly favor the interpretation that the contribution of EKLF in the inhibition of megakaryocytic differentiation is at least partially dependent on the repression of the *Fli-1* gene expression.

While this manuscript was under revision, a very interesting study[51] based on inducible EKLF overexpression in ES cells and analysis of *Eklf* -/- ES cells also concluded that EKLF represses megakaryocytic differentiation. The present study not only strengthens this conclusion but further establishes that this negative control of megakaryopoietic differentiation occurs at the benefit of erythropoietic differentiation. Both studies demonstrate that, in normal progenitors, the negative control of megakaryocytic differentiation occurs at least it part through the *Fli-1* gene repression. Understanding how EKLF represses the expression of the *Fli-1* gene is thus of crucial importance.

Previous studies have shown that EKLF displays two different repressor domains : the zinc fingers domain which can recruit Sin3A and HDAC1 [48,52] and more recently lysine 74 which when sumoylated can recruit the Mi-2β component of the NuRD repressor[53]. Interestingly, unlike wild type EKLF, a K74R EKLF mutant which cannot be sumoylated cannot inhibit megakaryocytic differentiation when it is overexpressed under the control of the PF4-gene promoter in transgenic mice [53]. Based on the detection of few amounts of EKLF on the *Fli-1* gene promoter in embryonic bodies derived from ES cells, a simple model could be proposed by which EKLF would bind to conserved EKLF putative DNA binding elements and then recruit the above repressor complexes [51,53]. Several new results obtained in the present study question this simple model. First, we found that the activation of *Fli-1* gene and FLI-1 target genes induced by EKLF knockdown in MEL cells is associated with a much stronger increase in FLI-1 occupancy at their promoters than that expected from the very slight increase of FLI-1 protein levels in the nucleus (Figure 3 and S2). This thus indicates that EKLF may be also involved in the inhibition the FLI-1 recruitment to its target promoters. Furthermore, we found that a zinc fingers mutant of EKLF which is unable to bind DNA[48] is still able to repress FLI-1-dependent activation of megakaryocytic genes in K562 cells (Figure S3) thus implying that if EKLF recruitment is involved in the repression of FLI-1 target genes this recruitment should be indirect. It is known that FLI-1 directly interacts with GATA-1 allowing their cooperative DNA binding and synergistic activation of megakaryocytic genes[24,54]. Symmetrically, EKLF has also been shown to interact and to cooperate with GATA-1 in the synergistic activation of some erythrocytic promoters[55]. Taking into account the striking opposite regulation of erythrocytic *versus* megakaryocytic genes in response to EKLF knockdown, it is tempting to speculate that FLI-1 and EKLF might compete with limiting amounts of common cofactors like GATA-1 for the formation of mutually exclusive multi-protein complexes able to activate either erythrocytic or megakaryocytic promoters. Our finding that EKLF knockdown is associated with a

concomitant increase of both FLI-1 and GATA-1 recruitment at the *Gp1bα* promoter would be in agreement with this possibility. Another alternative but non exclusive possibility could be that physical association between Fli-1 and EKLF [20] precludes the formation of these mutually exclusive positive complexes. Whatever are the exact mechanisms by which EKLF represses the commitment toward megakaryocytic lineage, this study already strongly suggests that this repression is mediated at least partially by the inhibition of FLI-1 protein function.

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Authorship :

FB designed and performed the experiments, interpreted the data and assisted with the paper; GJ performed experiments and interpreted the data, NC performed experiments and interpreted the data, DB assisted experiments on human progenitors, FL designed and assisted experiments on human progenitors and interpreted the data. BG interpreted the data and assisted with the writting of the paper. WV made valuable suggestions on the design of experiments and FM designed the experiments, interpreted the data and wrote the paper.

Conflicts of interest disclosure : The authors declare no competing financial interests.

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Legends to figures

Figure 1 :

Endogenous EKLF activates erythrocytic and represses megakaryocytic gene expression in MEL cells. 4D7 and 2M12 are two different clones derived from MEL cells which has been engineered to allow inducible expression of two different shRNA directed against *Eklf* mRNA (see methods). 4D7 and 2M12 cells were grown for 2 days in the presence of 2 µg/mL doxycycline to induce *Eklf* shRNA expression and then for 2 days in the presence of 5 mM HMBA (still in the presence of doxycycline) to induce their differentiation. 4D7 and 2M12 cells grown in the same conditions but without doxycycline were used as control. A : Western blot analysis of EKLF protein in 4D7 (left part) or 2M12 cells (right part) treated in the presence (lanes 2) or absence (lane 1) of doxycycline. Asterisk indicates unspecific band. Actin protein is shown as loading control. Percentages indicate relative levels of EKLF proteins (EKLF/Actin ratios) estimated by densitometry. B : Doxycycline-induced changes of erythrocytic (lanes 1-3) and megakaryocytic (lanes 4-7) mRNAs gene levels in 4D7 cells and 2M12 cells. Relative levels of each mRNA have been determined by quantitative RT-PCR using *Hprt* mRNA as a normalization reference. Final results are expressed as fold changes induced by doxycycline (means and standard deviations from 4 independent experiments).

Figure 2 :

EKLF knockdown increases RNA polymerase II occupancy and acetylation of histone H3 at the Gp1b and GpIIIa megakaryocytic gene promoters. 4D7 cells were treated for two days with (black bars) or without doxycycline (white bars) to knockdown EKLF followed by two days in presence of HMBA to induce their differentiation as described in Figure 1. Chromatin immunoprecipitation analyses were then performed using either control anti HA antibody or specific antibodies directed against RNA-Polymerase II (A) or acetylated histone H3 (B). Immunoprecipitated DNA were quantified by real time PCR using specific primers corresponding to the promoters or an internal position of *GpIX* and *Gp1b* megakaryocytic genes as well as to the silent *myoD* gene. Results are expressed as relative proportions of immunoprecipitated DNA (ratios of immunoprecipitated *versus* input DNA) standardized to the ratio obtained for the *GpIX* promoter in untreated cells (means and standard deviations from three independent cultures). Significant ($p<0.05$; paired Student's test) doxycycline effects are indicated by asterisks.

Figure 3 :

EKLF knockdown increases FLI-1 occupancy at the Gp1b, GpIX and Fli-1 megakaryocytic gene promoters. ChIP experiments were performed as described in Figure 2 (the same chromatin samples were used) using either anti FLI-1 (A) or anti GATA-1 (B) antibodies. Results are expressed as relative proportions of immunoprecipitated DNA (ratios of immunoprecipitated *versus* input DNA) standardized to the ratio obtained for the *Gp1b* promoter in untreated cells (means and standard deviations from three independent cultures). Significant ($p<0.05$; paired Student's test) doxycycline effects are indicated by asterisks (NS : non significant). Insert corresponds to the Western blot analysis of FLI-1, EKLF, GATA-1 and GRB2 present in total protein extracts from the 4D7 cells used for the ChIP experiments (stars correspond to unspecific bands). Percentages in brackets indicate the change of FLI-1, EKLF and GATA-1 levels induced by doxycycline as estimated by densitometry (relative values standardized to GRB2 and expressed as percentage of values without doxycycline).

Figure 4 :

EKLF knockdown enhances the megakaryocytic differentiation output of human CD34+ progenitors and decreases their expression of the known erythrocytic target gene Gpa. Human cord blood $CD34^+$ cells were amplified for three days in E/MK medium, infected for three hours with either *Eklf* shRNA or control SCR shRNA lentivirus and grown for further 7 days in E/MK medium as described in methods. Figure shows the analysis of GFP positive cells performed 7 days post-infection (typical results from three independent experiments). A : Western blot analysis showing the drastic reduction of EKLF protein level in purified GFP^+ cells infected with *Eklf* shRNA lentivirus (lane 2) compared to control shRNA lentivirus (lane 1). HSC70 protein is shown as loading control. B : FACS analysis of GPA expression in GFP^+ cells. Histograms show that most of the GFP^+ cells infected with *Eklf* shRNA lentivirus still express GPA (black line, gate M1) but at lower level than cells infected with control shRNA lentivirus (filled histogram, gate M1). Control isotype histogram is shown as dotted line. The percentages of GPA positive cells as well as the mean geometric fluorescence of GPA (in brackets) for each population are indicated. C : Typical FACS analysis showing a two fold increase in the percentage of $CD41^+CD42^+$ differentiated megakaryocytic cells among GFP^+ cells infected with the *Eklf* shRNA lentivirus compared to that infected with the control shRNA lentivirus (left panel). Histogram (right panel) shows the mean and standard deviation of the fold increase in megakaryocytic cells obtained in three independent experiments (p value of 0.05 in paired Student's test). D : Comparison of erythrocytic (lanes 1

and 2) and megakaryocytic (lanes 3-5) genes mRNA levels in GFP^+ cells infected with *Eklf* or control shRNA lentivirus. Relative levels of each mRNA have been determined by quantitative RT-PCR using *Hprt* as a normalization reference. Final results are expressed as fold changes induced by EKLF knockdown (shEKLF/shSCR ratios; means and standard deviations from 3 independent experiments).

Figure 5 :

EKLF knockdown in normal human $CD34^+$ progenitors does not induce megakaryocytic genes expression in late differentiated erythrocytic cells. Human cord blood $CD34^+$ cells were amplified for three days in medium favoring erythrocytic progenitors amplification, infected with either *Eklf* shRNA (black boxes) or control SCR shRNA lentivirus (white boxes) and grown for further 3 days in medium favoring terminal erythroid differentiation as described in methods. A : western blot analysis of EKLF performed on the whole GFP^+ infected cell populations. B : Quantitative RT-PCR analyses of erythrocytic (*Eklf, Gpa and β-globin*) and megakaryocytic (*GpIIIa* and *GpIX*) mRNA levels in total GFP^+ infected cells (lanes 1, 2) and in purified late erythrocytic $GPA^+CD36^+GFP^+$ infected cells (lanes 3, 4). Results are expressed as relative levels (normalized to *Hprt* mRNA) after standardization to the level determined in the whole cell population infected with control lentivirus (lane 1). Note that the levels of *GpIIIa* and *GpIX* mRNA observed in the whole cell population (lanes 1 and 2) were at least ten fold lower than that observed in the whole cell population obtained in the culture conditions favoring both erythrocytic and megakaryocytic differentiation in Figure 4.

Figure 6 :

EKLF knockdown in normal human progenitors enhances megakaryocytic differentiation at the expense of erythrocytic differentiation. Human cord blood $CD34^+$ cells were amplified for three days in E/MK medium, infected for three hours with either *Eklf* shRNA or control SCR shRNA lentiviruses. Immediately following infection, $CD36^+CD31^{Med}$ cells enriched in bipotent progenitors were sorted by FACS and reseeded for 7 days in E/MK as described in methods and supplementary Figure S1. Each well was inspected under fluorescent microscope to identify clones containing GFP^+ cells attesting successful infection and classified as megakaryocytic, erythrocytic or mixed clones based on light-microscope observation, FACS analysis of the expression of GPA and CD41 and benzidine staining. Erythrocytic clones were identified by a high number of small cells expressing GPA but not CD41 and containing benzidine positive cells; megakaryocytic clones were identified by a small number of large

cells expressing CD41 but not GPA and containing no benzidine positive cells; mixed clones were identified by an intermediate number of both types of cells. A : Typical FACS diagrams and light-microscope fields illustrating erythrocytic, megakaryocytic and mixed clones obtained. B : Histogram shows the percentages of these three different types of GFP^+ clones derived from cells infected with either *Eklf* shRNA (black bars) or control shRNA virus (white bars) (means and standard deviations from three independent experiments with 60-92 GFP^+ clones recorded for each condition). Statistically significant differences ($p<0.05$; paired Student's test) are indicated by asterisks (NS : non significant).

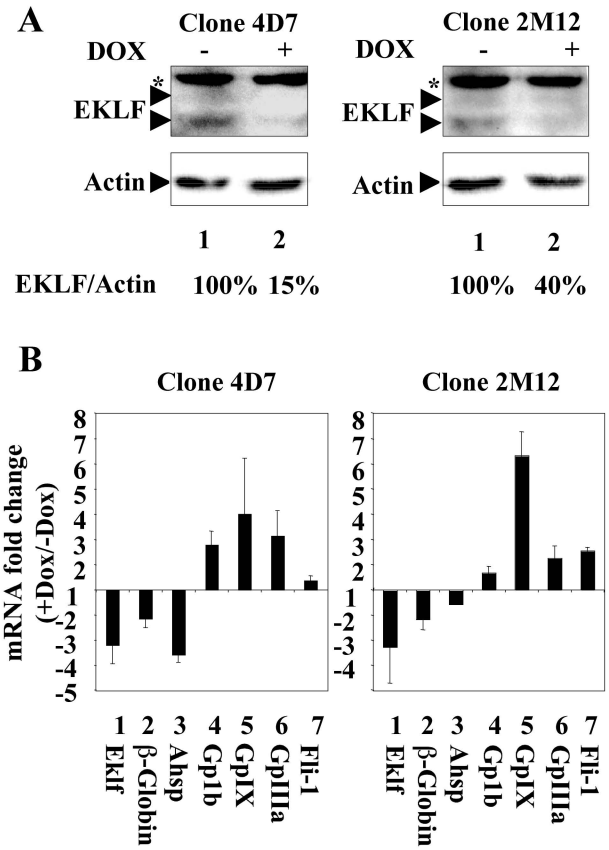

Figure 1

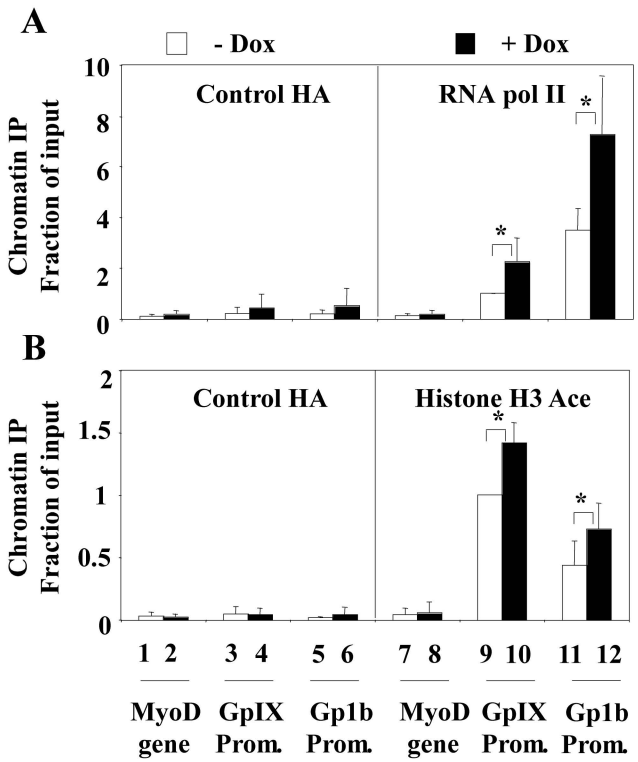

Figure 2

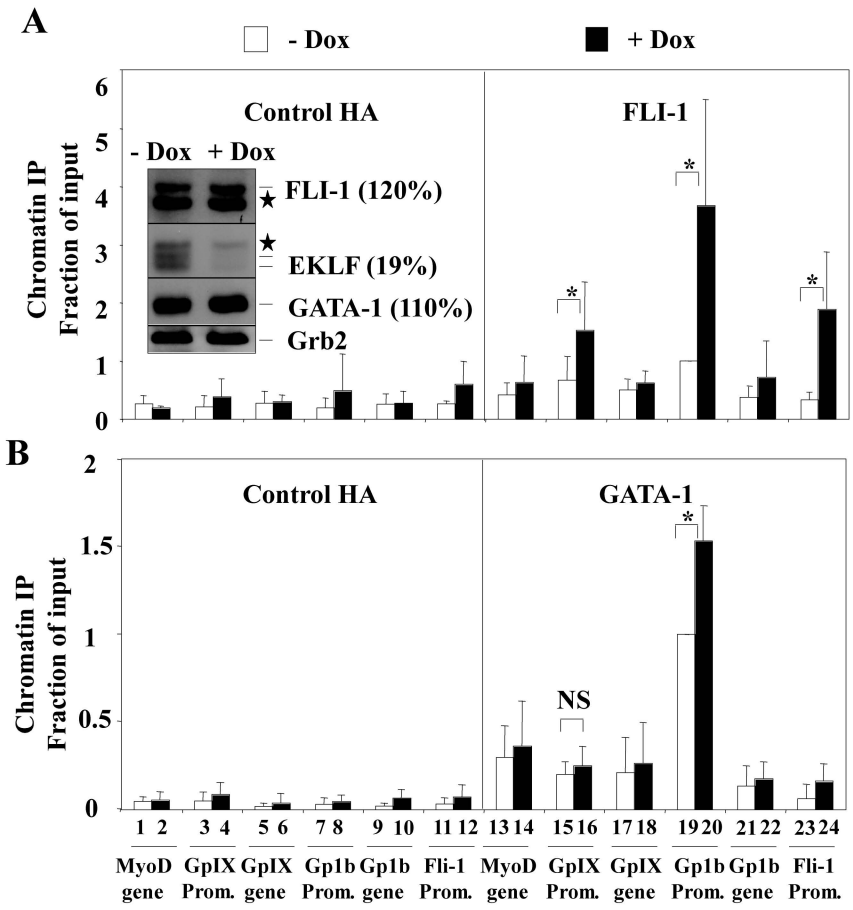

Figure 3

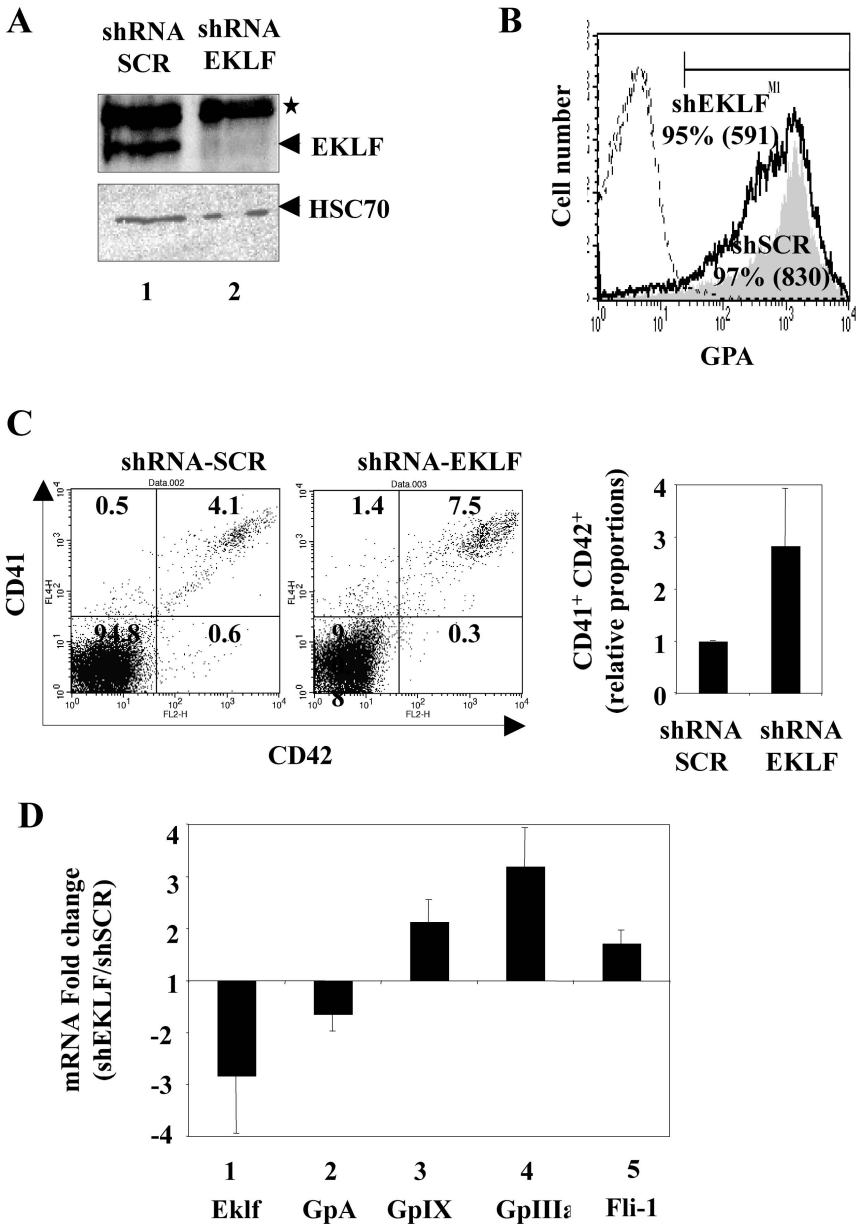

Figure 4

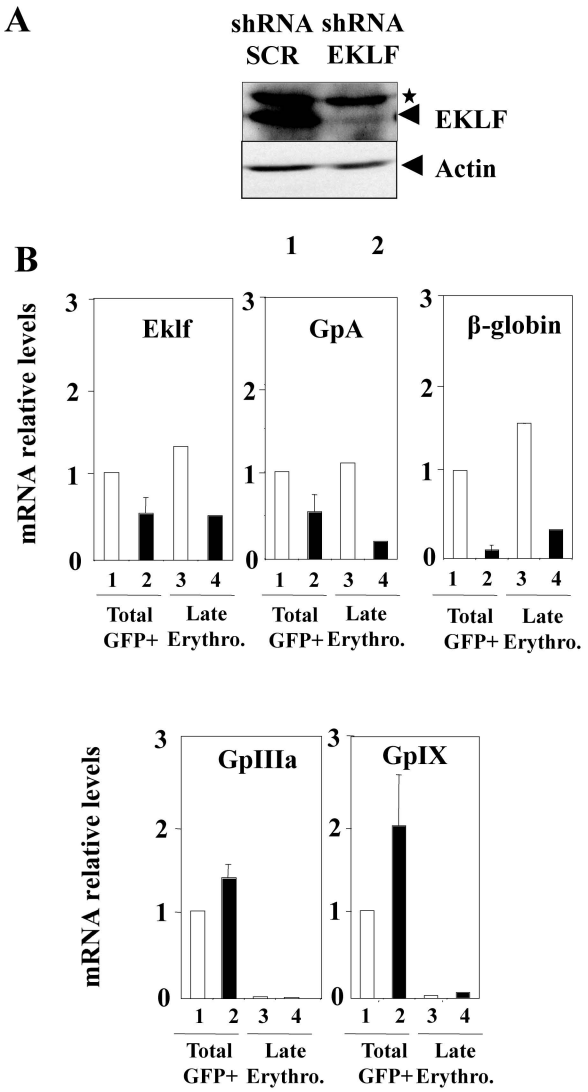

Figure 5

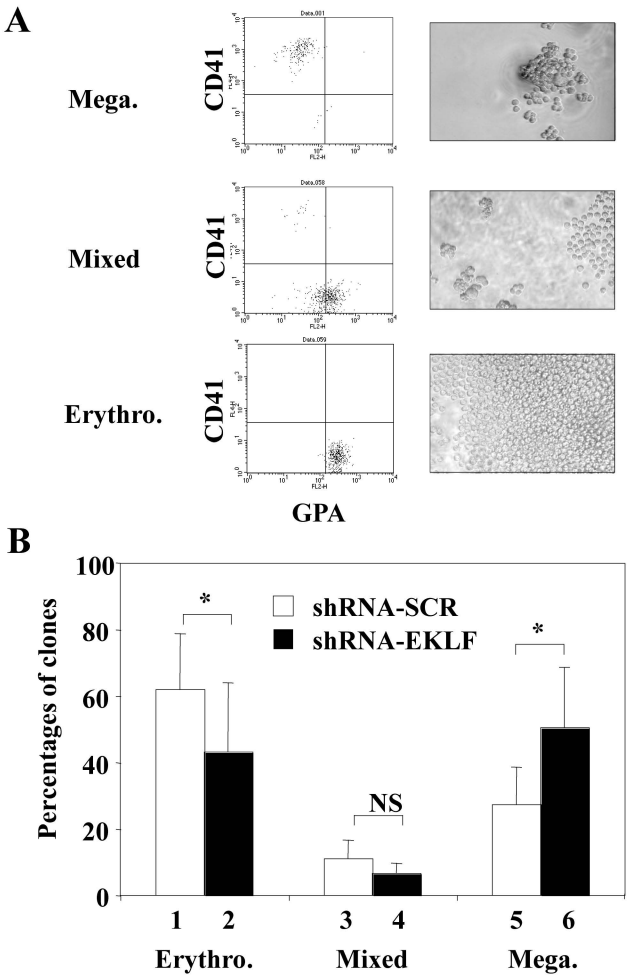

Figure 6